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Subcellular Sites Involved in Lipid Synthesis in *Saccharomyces cerevisiae*

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When the crude ribosomal fraction of *Saccharomyces cerevisiae* was separated into "light" and "heavy" fractions, fatty acid synthetase was concentrated in the former, whereas acetyl-Coenzyme A synthetase, fatty acid "desaturase," and squalene oxidocyclase were found in the latter. The "desaturase" sedimented with the ribosomal material and was not solubilized by low concentrations of sodium deoxycholate (DOC). The other two systems found in the "heavy" fraction sedimented with the membranes, but, upon solubilization of the membranes by DOC, these enzyme systems remained as particles.

The microsomal fraction of animal cells has been implicated in many of the reactions involved in the biosynthesis of lipids. Abraham and coworkers (1, 2) have shown that rat liver microsomes strongly stimulate the synthesis of fatty acids by rat liver supernatant preparations. In addition, microsomes have been shown to be able to convert malonyl-coenzyme A (CoA) to long-chain fatty acids (1, 9). The desaturation of fatty acids in rat liver homogenates has also been ascribed to microsomal enzymes (2, 10).

The synthesis of cholesterol in rat liver preparations was initially found to require both particulate and soluble fractions of the cell (6). Later, Tchen and Bloch (17) demonstrated that rat liver microsomes were involved in the cyclization of squalene to sterols. In a recent report (7), Chesterton suggested that the formation of cholesterol, lanosterol, and other intermediary sterols takes place in the microsomal fraction of rat liver preparations.

Investigations with yeasts have yielded results that, in many ways, resemble those obtained with animal systems. Thus, for example, the oxidative desaturation of fatty acids in *Saccharomyces cerevisiae* was attributed, by Bloomfield and Bloch (4), to a particulate fraction of these cells. The fatty acid synthetase of *S. cerevisiae* is also known to be a particulate enzyme system (13, 16). In the biosynthesis of nonsaponifiable lipids, the cyclization of squalene to sterols has been reported to be particle bound (11, 12). In some of these studies with yeasts, there have been vague references to a "microsomal" fraction, since the enzymes referred to were generally observed in preparations from which the mitochondria had been previously removed. However, there is little evidence available for a true microsomal system in *S. cerevisiae* (H. P. Klein et al., J. Bacteriol., *in press*).

In our studies of lipid synthesis in *S. cerevisiae*, we have found that the crude ribosomal fraction is capable of incorporating acetate into both saturated and unsaturated fatty acids (1, 13), thus indicating the presence in this material of acetyl-CoA synthetase, acetyl-CoA carboxylase, fatty acid synthetase, and fatty acid "desaturase," the enzyme catalyzing the oxidative desaturation of long-chain saturated fatty acids. In addition, these crude particles were also required for the conversion of squalene to sterols (12), thus indicating the presence of the squalene cyclizing system. Since the crude ribosomal fraction contains membranes as well as a variety of discrete particles (H. P. Klein et al., J. Bacteriol, *in press*), and since at least partial resolution of some of these components can be achieved (12, 13; H. P. Klein et al., J. Bacteriol., *in press*), it seemed of interest to correlate some of the enzyme systems involved in lipid synthesis with specific subcellular structures.

MATERIALS AND METHODS

Organism. S. cerevisiae (strain LK2G12) was grown as described previously (11, 12). Methods for disrupting cells and for obtaining the crude ribosomal fraction have been already given (11). In some experiments in which the membranous portion of this fraction was to be labeled, cells were grown with ^{14}C-choline (H. P. Klein et al., J. Bacteriol., *in press*). Pro-

cedures to obtain the "light and "heavy" fractions of the crude ribosomal fraction are described elsewhere (14; H. P. Klein, *in press*).

Assay of acetyl-CoA synthetase. The activity of this enzyme was measured simply by the incorporation of acetate-*1*-^{14}C into nonsaponifiable lipids. In the absence of the crude ribosomal fraction, the soluble supernatant fluid had a very limited capacity to incorporate acetate not only into fatty acids but also into nonsaponifiable lipids. Accordingly, any stimulation of acetate incorporation into nonsaponifiable lipids in the presence of soluble supernatant fluid and suitable cofactors was used as a measure of acetyl-CoA synthetase activity. Such determinations have been found to correlate with direct assays for this enzyme, by use of the hydroxylamine trapping technique of Berg (3).

Assay of fatty acid synthetase. This system was assayed directly by the incorporation of malonyl-CoA-*1,3*-^{14}C into fatty acids (13, 16).

Assay of "desaturase." The activity of this system was determined, through use of gas chromatography, as the ratio of unsaturated to saturated fatty acids produced during any specific experiment in which ^{14}C-acetate or ^{14}C-malonyl-CoA was the substrate. In the absence of the appropriate particulate fractions, this ratio was generally 0.2 to 0.4. This ratio increased 10- to 20-fold in the presence of particles containing the desaturating system.

Assay of squalene oxidocyclase. This system also was assayed indirectly. After incubation of the test fractions with ^{14}C-acetate, the nonsaponifiable lipids were extracted and chromatographed on alumina columns (11). Enzyme preparations deficient in this enzyme system yielded mainly squalene, whereas the addition of the appropriate particles resulted in a shift in the major product to sterols (11, 12). [Recently, evidence has accumulated that the squalene oxidocyclase system may contain two enzymes, a squalene oxidase and a cyclizing enzyme (8). In the experiments reported here, both enzymes presumably are present in the particle fractions tested, since we have shown earlier (11) that the crude particles alone can convert squalene to sterols.]

Chemical methods. Procedures for the incubation of extracts, subsequent hydrolysis, and extraction of lipids are described elsewhere (11, 12, 18). Acetate-*1*-^{14}C was purchased from New England Nuclear Corp., Boston, Mass. The method of preparing radioactive malonyl-CoA was a modification of the one described by Lynen (15). The resulting procedure, at least in our hands, yielded better results and is therefore described here in detail.

S-malonyl-*N*-capryl-cysteamine was prepared by stirring a mixture of 193 mg (1.85 mmoles) of dry malonic acid, 0.14 ml (1.93 mmole) of thionyl chloride (Eastman Chemical Products, Inc., Kingsport, Tenn., white label), and 5 ml of dry ether for 15 hr at 45 to 48 C. The solvent was removed in vacuo at 25 C, and the residue was dissolved in 1 ml of ethyl acetate. *N*-capryl-cysteamine (475 mg, 2.34 mmole) was added, and the mixture was stirred under vacuum for 2 hr at room temperature with a magnetic stirrer. The mixture was then partitioned between 6 ml of ethyl acetate and 1 ml of water. The ethyl acetate was extracted with two 1-ml portions of saturated $NaHCO_3$, and the extract was cooled to 0 C and was adjusted to *p*H 1 to 2 with 6 N HCl. The white precipitate was collected by filtration, washed with water, and dried in vacuo, leaving 235 mg of white crystals, mp 67 to 68 C. (The infrared spectrum of this product was identical to authentic material, mp 70 to 71 C.)

N-caprylcysteamine was made by refluxing a mixture of 5.0 g of *N,N'*-dicaprylcysteamine (15), 5.0 g of sodium borohydride, and 250 ml of isopropanol for 2 hr. The solvent was evaporated in vacuo, and the residue was treated with 100 ml of water. After filtration, the alkaline filtrate was taken to *p*H 1 to 2 to precipitate the mercaptan. The white crystals were collected, washed with water, and dried to leave 4.5 g, mp 48 to 49 C [90% yield; mp was 50–51 C according to Lynen (16)].

Gas chromatography was carried out by use of procedures given earlier (12, 18). Radioactivity determinations were made with the Packard scintillation counter (model 500C) as previously reported (12). Estimations of protein (Lowry method) and of ribonucleic acid (RNA) (orcinol method) are referred to in other publications (11–14; H. P. Klein et al., J. Bacteriol., *in press.*)

RESULTS

Enzymatic activities associated with the "light" and "heavy" fractions of the crude ribosomal material. In a recent report (14), it was shown that a "light" fraction could be obtained from the crude ribosomal material by washing the latter twice in tris(hydroxymethyl)aminomethane (Tris)-Mg buffer and then sedimenting the combined washings at 100,000 × *g* for 90 min. The resultant "light" particles were found to contain virtually no membrane material, to have a protein to RNA ratio of about 12, and to be rich in the fatty acid synthetase complex (14). For the experiment summarized in Table 1, cells were grown in the presence of isotopically labeled choline, and, after separation of the crude ribosomal material into "light" and "heavy" fractions, the two particulate preparations were assayed for labeled phospholipid. The "light" fraction contained negligible amounts of radioactivity, indicating the virtual absence of membranous contamination. The different fractions were also assayed for fatty acid synthetase, acetyl-CoA synthetase, "desaturase," and squalene oxidocyclase. The "light" fraction had an enriched fatty acid synthetase activity, low acetyl-CoA synthetase activity, and essentially no "desaturase" or squalene oxidocyclase activity. On the other hand, the "heavy" fraction retained about 65% of the initial membranous component (as measured by the radioactivity of the extracted lipids). The specific activity of the fatty acid synthetase in this fraction fell to about half that of the original particles, whereas the other three

TABLE 1. *Lipogenic activities of the "light" and "heavy" fractions*[a]

Fraction	Phospholipid (total counts/min)[b]	Acetyl-CoA synthetase[c]	Fatty acid synthetase[d]	"Desaturase"[e]	Squatone oxidocyclase[f]
Crude ribosomal particles	1.4×10^8	32,100	57,695	4.2	15
"Light" fraction	0.006×10^8	7,735	154,620	0.3	0
"Heavy" fraction	0.9×10^8	25,655	32,395	4.1	8

[a] Cells were grown in 10 liters of medium containing ^{14}C-choline (4.5×10^7 counts per min, 4.3 μmoles per liter). A 72-g (wet weight) amount was resuspended after washing to a total of 144 ml in Tris (0.002 M)-$MgCl_2$ (0.002 M) buffer (*pH* 7.6), and was broken in a French pressure cell (12). Of the resulting homogenate, 85 ml was further fractionated into crude ribosomal particles. A portion of the latter was separated into "light" and "heavy" fractions (13). Suspensions of particles contained (milligrams of protein per milliliter): crude ribosomes, 21; "light" fraction, 2.6; and "heavy" fraction, 18. Of each fraction, 0.2 ml was used for enzyme assays.

[b] Measured by ^{14}C-choline incorporation into extracted lipids (15).

[c] Measured by ^{14}C-acetate incorporation into nonsaponifiable lipids, in presence of soluble supernatant fluid (13). Expressed in counts per minute per milligram of protein.

[d] Measured by ^{14}C-malonyl-CoA incorporation into fatty acids (13). Expressed in counts per minute per milligram of protein.

[e] Ratio of unsaturated to saturated fatty acids (12).

[f] Expressed as a percentage of sterols. Measured by increase of sterols in presence of soluble supernatant fluid. Values reported are percentage of nonsaponifiable lipids that chromatograph on alumina columns as sterols above that of the supernatant fluid alone (11).

enzyme systems did not appear to have been lost from this fraction by subjecting the crude ribosomal fraction to these procedures.

Since the "heavy" fraction is known (H. P. Klein et al., J. Bacteriol., *in press*) to contain the bulk of ribosomes as well as membranes, it is evident that those activities sedimenting with the "heavy" fraction (Table 1) could be bound either to ribosomes or to membranes, or could represent other relatively heavy particulate components. Since previous experiments (H. P. Klein et al., *in press*) demonstrated that the membranous components sedimented somewhat more rapidly than did the ribosomes, a preparation of crude ribosomal material labeled with ^{14}C-choline was subjected to centrifugation at 100,000 × *g* for increasing periods of time, and the resulting supernatant fluids were assayed for the presence of membranes (i e., radioactivity extractable as phospholipid), ribosomes, and for the enzymes discussed above. The membranous material sedimented more rapidly than the ribosomes (Table 2), whereas the fatty acid synthetase remained suspended during the course of the experiment. Of the three enzyme activities associated with the "heavy"fraction (Table 1), the acetyl-CoA synthetase and the squalene oxidocyclase sedimented more rapidly than did the "desaturase," which behaved as if it might be bound to ribosomes.

Effects of sodium deoxycholate (DOC). In earlier work (H. P. Klein et al., *in press*), it was reported that treatment of the "heavy" fraction by 1% DOC essentially removed all the membranous elements from this fraction. Accordingly, such treatment was carried out in connection with these studies to determine whether "solubilization" of the membranes would simultaneously lead to a release of any of the bound enzymes into a soluble form. At the outset, it was noted that 1% DOC invariably destroyed "desaturase" activity.

TABLE 2. *Effect of centrifugation on subcellular components and on lipogenic activities*[a]

Component measured	Percentage of initial values after centrifugation (at 100,000 × *g*) of			
	0 min	1 min	5 min	15 min
Fatty acid synthetase[b]	100	100	100	100
Ribosomes[c]	100	83	78	64
"Desaturase"[b]	100	90	78	50
Membranes[b]	100	68	48	30
Squalene oxidocyclase[b]	100	67	40	25
Acetyl-CoA synthetase[b]	100	41	32	20

[a] Crude ribosomal particles (1,080 mg of protein) were obtained and resuspended to 54 ml as in Table 1. Samples of 9 ml were centrifuged at 100,000 × *g* for the times indicated, and the resulting supernatant fluids were then subjected to analysis. Of each suspension, 0.2 ml was used for enzyme assays. Protein concentrations were (milligrams per milliliter): at 0 min, 20; 1 min, 18; 5 min, 17; 15 min, 14.

[b] Measured as in Table 1.

[c] Measured as bound RNA.

However, when lower final concentrations of this compound were used, reasonable "solubilization" of bound phospholipids was achieved without any impairment of the activity of this enzyme. When a preparation of the "heavy" fraction, previously labeled with C^{14}-choline, was treated with 0.1% DOC, about 80% of the lipid-extractable radioactivity was lost from the particles sedimented after such treatment (Table 3). Nevertheless, the remaining particles were fully active in catalyzing the desaturation of fatty acids. When the DOC concentration was raised to 0.2%, there was a further reduction in the amount of membranous material remaining in the sedimentable particles, but without an effect on the "desaturase" activity of this material.

Table 4 shows the effect of treating the crude ribosomal fraction with 1% DOC on the localization of acetyl-CoA synthetase and squalene oxidocyclase. In this experiment, one sample of crude ribosomal particles was separated into "heavy" and "light" fractions which were then assayed for these two enzymes and for membranes. Another sample of crude ribosomal material was first treated with 1% DOC and then subjected to the identical treatment. Without DOC treatment (Table 4), the two enzymes sedimented with the membranes in the "heavy" fraction as was the case in the experiment summarized in Table 1. After DOC treatment, most of the membranous material was lost from the two sedimentable particulate fractions. DOC treatment also effectively removed the two enzymes from the "heavy" fraction. However, in contrast to the membranes, these enzymes were not solubilized. Instead, they were now recovered in the "light" fraction.

DISCUSSION

Information about the probable intracellular location of four of the enzyme systems that operate in the biosynthesis of lipids has been obtained in these studies. The fatty acid synthetase particles, by virtue of the fact that they can be separated readily from both the membranous and the ribosomal fractions of *S. cerevisiae*, would

TABLE 3. *Effect of sodium deoxycholate (DOC) on the fatty acid "desaturase" activity of the "heavy" fraction*[a]

DOC used (%)	Membranes[b]	"Desaturase"[c]
0	5.8×10^5	4.8
0.1	1.1×10^5	5.6
0.2	0.7×10^5	5.4
1.0	0.03×10^5	0.3

[a] Cells were grown and processed as in Table 1. A suspension of crude ribosomal particles in Tris-Mg buffer was divided into four samples. DOC was then added to the suspensions to yield the final concentrations shown. Immediately after mixing, the suspensions were centrifuged to yield "light" and "heavy" fractions. The "heavy" fraction derived from each suspension was then assayed for "desaturase" activity and for radioactive lipids.

[b] Measured by ^{14}C-choline incorporation into lipids. Expressed in counts per minute.

[c] Determined by gas chromatography; ratio of unsaturated to saturated fatty acids.

TABLE 4. *Effect of sodium deoxycholate (DOC) on acetyl-CoA synthetase and squalene oxidocyclase activities*[a]

Component measured	Treatment			
	No DOC		After 1% DOC	
	In "heavy" fraction	In "light" fraction	In "heavy" fraction	In "light" fraction
Membranes[b]	6.0×10^5	0.1×10^5	0.3×10^5	0.3×10^5
Acetyl-CoA synthetase[c]	30,670	7,915	2,880	62,300
Squalene oxidocyclase[d]	24	0	0	39

[a] A crude ribosomal suspension, containing 12 mg of protein per ml, was separated into two portions. One was treated with 1% DOC (final concentration), and the other was kept in buffer. Both preparations were then centrifuged to yield "light" and "heavy" fractions. The latter were resuspended in Tris-Mg buffer and analyzed as indicated. Without DOC treatment, protein concentrations were (milligrams per milliliter): "heavy" fraction, 15; "light" fraction, 0.9. After DOC treatment, the same concentrations were: "heavy" fraction, 9; "light" fraction, 3.9. Samples of 0.2 ml of suspended particles were used for enzyme assays.

[b] Measured as in Table 1. Figures refer to total counts per minute in fractions.

[c] Measured as in Table 1. Figures refer to counts per minute per milligram protein.

[d] Measured as in Table 1.

appear to be freely suspended in the intracellular fluid. Essentially all of the fatty acid synthetase activity of crude homogenates of this organism can be accounted for by these particles. No activity can be demonstrated in washed mitochondrial preparations of this organism, despite the fact that the mitochondrial fraction accounts for approximately half the fatty acid-containing material present in crude homogenates. It is therefore puzzling to account for the fatty acid content of these organelles. It would appear that mitochondrial formation must involve the synthesis of lipids outside the mitochondria and that these substances, perhaps as preformed subunits, are built into new mitochondria. It is also possible that in mitochondria fatty acid synthesis proceeds by way of a mechanism not involving malonyl-CoA (i.e., not involving fatty acid synthetase). However, we have been unable to demonstrate the incorporation of acetate or of acetyl-CoA into fatty acids by mitochondrial preparations.

The "desaturase" system parallels the sedimentation behavior of the ribosomes. This system is not solubilized under conditions which destroy the membranes. Accordingly, these results suggest that this system is firmly bound to ribosomes. However, an alternate explanation is that this system exists as a separate particle—one that is considerably heavier than the fatty acid synthetase and that coincidentally sediments with the ribosomes during differential centrifugation. The fact that "desaturase" activity is destroyed by high concentrations of DOC (which have no effect on the other systems tested here) suggests, in fact, that this enzyme system is a lipoprotein complex.

Acetyl-CoA synthetase and squalene oxidocyclase, like the "desaturase," are found in the "heavy" fraction of the crude ribosomal material. However, these two enzymes sediment somewhat more rapidly than do the ribosomes, approximating the sedimentation behavior of the membranes. As was shown in Table 4, however, solubilization of the membranes with DOC was accompanied by a shift in the sedimentation characteristics of these two systems such that they now appear to be "light" particles. This behavior could mean that these enzymes are attached to, or are integral parts of, the internal membrane systems of this organism. In this regard, it is of interest to note the recent work with freeze-etched preparations in electron microscopy; for example, Branton (5) has described small particles, approximately 85 A in diameter, in exposed sections of inner membrane faces from a variety of cells, including yeasts. He has suggested that such particles, occurring at regular intervals in the membranes, are substructures of this cellular element.

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